Enterovirus 71 Infection of Human Dendritic Cells

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Enterovirus 71 (EV71) causes death and long-term neurologic sequelae in hundreds of thousands of young children, but its pathogenesis remains elusive. Dendritic cells (DCs) play a crucial role in antiviral immunity by functioning as professional antigen-presenting cells to prime T cells and by secreting cytokines to modulate immune responses. Here, we show that EV71 productively infected human immature DCs and expressed viral antigen in DCs. EV71 entry into DCs was partially mediated by DC-SIGN. Further analyses revealed that EV71 increased the viability, activation, release of cytokines, interleukin-6, interleukin-12, and tumor necrosis factor-α in DCs. Moreover, EV71 enabled DCs to stimulate T-cell proliferation. Collectively, these findings suggest that EV71 infection of human DCs *in vivo* is very likely to elicit protective immunity, because in infected mice, both T cells and IL-6 function to reduce mortality. Exp Biol Med 234:1166–1173, 2009

Key words: enterovirus 71; dendritic cells, and infection

Introduction

Enterovirus 71 (EV71), a neurotropic picornavirus, infects the human central nervous system and induces fatal neurological manifestations. Severe symptoms, such as brainstem encephalitis and cardiopulmonary complications, often cause death or long-term neurological sequelae, especially in young children (1–5).

In the past decade, the Asia-Pacific region had more frequent and widespread EV71 outbreaks, which are estimated to have infected millions of children and have caused death and long-term neurological sequelae in hundreds of thousands of young children (6). The largest and most fatal outbreak occurred in Taiwan in 1998, with 405 severe cases and 78 deaths reported (2). Since then, EV71 infection has become endemic in Taiwan and caused >40, >40, and 14 deaths in 2000, 2001, and 2008, respectively (7). There are no effective vaccines or antiviral therapies currently available to control fatal outbreaks due in part to the lack of understanding of viral pathogenesis.

Some clinical studies found lower cellular immunity in patients with brainstem encephalitis and pulmonary edema and proposed that the reduced cellular immunity may cause severe symptoms by resulting in viral dissemination (8, 9). A combination of interferon (IFN)-α, an antiviral agent which has been shown to protect mice from EV71 infection (10), and IFN-γ, which functions to enhance cellular immunity, is therefore recommended for the treatment of severe cases (8). However, some clinical studies revealed that besides young age, elevated white blood cells were associated with unfavorable outcomes (11, 12). High levels of white blood cell counts in blood and cerebrospinal fluid with a predominance of lymphocytes were found in patients with fatal sequelae (11–13). In addition, autopsy studies observed severe inflammatory infiltrate mainly composed of mononuclear cells in the central nervous system of expired patients (5). Immunopathology is therefore suspected to cause neurologic symptoms, and anti-inflammatory agents, such as corticosteroids, have been used to treat severe cases (3, 14). Besides elevated white blood cells, high levels of cytokines, such as interleukin-6 (IL-6), tumor necrosis factor (TNF)-α), and IFN-γ, were detected in the sera and cerebrospinal fluid of patients with fatal symptoms (9, 15–18). Cytokine storm is thus proposed to cause severe symptoms, so intravenous immunoglobulin and pentoxifylline, which suppress cytokine production, have been used or suggested to treat severe cases (9, 16–18).

Currently, the role of host immunity in EV71 infection is controversial. Our study investigating EV71 pathogenesis using a mouse model found high mortality rates and tissue

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viral loads in infected mice deficient in $CD4^+$ T, $CD8^+$ T, or B cells (19). Most importantly, dendritic cells (DCs) from the brains of infected, but not of uninfected, mice expressed viral antigen and primed T cells efficiently. Moreover, an increase of cellular immunity, especially DCs, by the pretreatment of a hematopoietic growth factor, Flt3 ligand, protected mice from EV71 infection by reducing tissue viral loads. Thus, EV71 infection enhances mouse DCs to elicit protective immune response, although the detailed mechanism regarding how the virus interacts with DCs is not clear. There is no report on EV71 infection of human DCs available to validate our mouse study or justify our recommendations for patients based on our mouse study. Previous studies investigating the infection of human DCs by other enteroviruses, poliovirus, echovirus, and coxsackievirus B are inconsistent with our mouse study, because they showed that these enteroviruses impaired the capacity of DCs to prime T cells by reducing the viability and activation of DCs (20, 21). In the present study, we therefore investigated EV71 infection of human DCs.

Materials and Methods

Generation of Human Immature DCs. Peripheral venous blood obtained from healthy blood donors was kindly provided by Tainan Blood Center. Peripheral blood mononuclear cells (PBMC) were purified from the peripheral blood by Ficoll-Hypaque gradient centrifugation. Monocytes were isolated from PBMC by adhesion to plastic dishes for more than 2 hrs at 37°C as previously described (22). Immature DCs were generated from monocytes by culturing in PRMI 1640 medium containing 10% fetal bovine serum, 1000 U/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF), 500 U/ml of IL-4 (R&D Systems, Minnesota, USA), and antibiotics for 7 days. Medium was changed 2, 4, and 6 days after culturing.

Flow Cytometry. DCs were stained with phycoerythrin-conjugated control antibodies (Abs) or Abs (BD Biosciences, New Jersey, USA) against human antigens, CD1a (clone HI149), CD14 (clone M5E2), CD80 (clone L307.4), CD83 (clone HB15e), CD86 (clone IT2.2), MHC I (HLA-A, B, C; clone G46–2.6), or MHC II (HLA-DR; clone TÜ36) for 45 mins on ice. The stained cells were analyzed by a FACSCalibur (BD Biosciences) and WinMDI software.

Cell and Virus. A human muscle (rhabdomyosarcoma, RD) cell line was maintained in medium according to the instructions of American Type Culture Collection. EV71 strain Tainan/4643/98 (GenBank accession number AF304458) isolated from a patient with encephalomyelitis in Taiwan in 1998 (23) was propagated and titrated on RD cells and used to infect DCs.

Infection of DCs by EV71. DCs were infected with EV71 at a multiplicity of infection (MOI) of 10 or 1 for 1.5 hrs at 37°C. Infected DCs were washed 2 times and cultured in RPMI medium. Culture supernatants were harvested 2–48 hrs after infection to determine viral titers by plaque assay on RD cell monolayers or 24 hrs after infection to measure cytokines, IL-6, IL-12, TNF-α, and IFN-γ by ELISA kits (R&D Systems) according to the manufacturer's protocols.

RT-PCR. Total RNA was isolated from mock- or EV71-infected DCs 24 hrs after infection and split into 2 portions. To detect positive-strand viral RNA genomes and β-actin transcripts, one portion of total RNA was annealed with 2.5 μM of the virus reverse primer, EV71–2 (5′-GTTATGTCTATGTCCCAGT T-3′; genome position 2762 to 2781) and β-actin reverse primer, β2 (5′-CAGGGTACATGGTGGTGCC-3′). To detect negative-strand viral RNA genomes and β-actin transcripts, the other portion of total RNA was annealed with the virus forward primer, EV71–1 (5′-GTGGCAGATGTGATTGAGAG-3′; genomic position 2450 to 2469) and primer β2. Mixtures were subjected to thermal cycling for 5 mins at 70°C and then cooled down to 4°C for the RNA to denature and anneal with primers. Half of each sample was added to a RT reaction mixture containing 40 U of RNase inhibitor, 0.6 mM deoxynucleotide triphosphates (dNTPs), 6 mM Mg^{2+}, and buffer with or without ImProm-II™ RT (Promega, Wisconsin, USA) and reverse transcribed for 5 mins at 25°C, 60 mins at 42°C, 15 mins at 72°C, and then cooled down to 4°C. To amplify viral cDNA, aliquots of cDNA were combined with a PCR mixture containing 2.5 U of *Taq* polymerase, buffer, 0.25 mM dNTPs, 1.5 mM Mg^{2+}, and 0.5 μM EV71–1 and EV71–2 primers and then subjected to thermocycles to amplify 332-bp DNA products. The PCR conditions were 94°C for 7 mins, 39 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min and then an additional 10 mins in the last cycle. To amplify β-actin cDNA, PCR was performed as previously described (22) using primers β2 and β1 (5′-AGCGGGAA ATCGTGCGTG-3′) to synthesize 309-bp DNA products. PCR products were separated by electrophoresis on agarose gels containing ethidium bromide.

Immunofluorescence Assay. DCs were harvested 24 hrs after infection, immobilized on slides by cytospin, fixed with 3.7% formaldehyde, permeabilized with acetone, and stained with a monoclonal Ab (mAb) against EV71 capsid protein VP1 (422–8D-4C-4D; Chemicon, Massachusetts, USA) or a control Ab (BD Biosciences). After being washed, slides were incubated with fluorescein isothiocyanate–conjugated anti-mouse immunoglobulin G (BD Biosciences) for 1 hr. After being washed, slides were stained with a phycoerythrin-conjugated anti-human CD1a Ab. Slides were viewed and photographed by a fluorescence microscope.

Ab Treatment. DCs were treated with a mAb against DC-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN; clone 120612, R&D Systems) or a control Ab 1 hr before infection with EV71. Infected cells were washed 2 times and cultured in medium for 24 hrs. Culture supernatants were harvested to determine viral titers.

Mixed-Lymphocyte Reaction. T cells were purified by depleting monocytes from PBMC by adhesion to plastic dishes for 2 hrs at 37°C, and then T cells were isolated from the

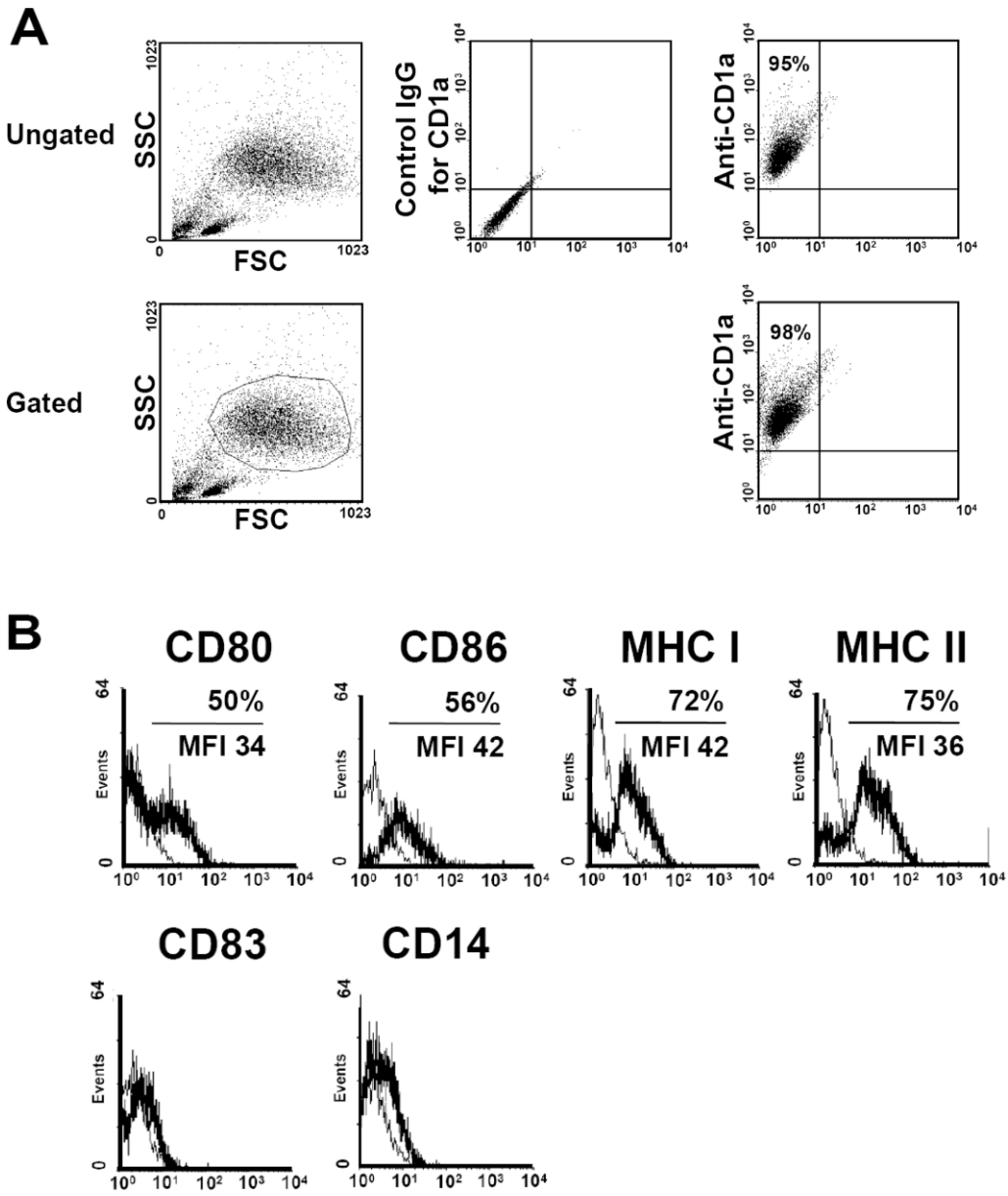

Figure 1. Flow cytometric analysis of human blood monocyte-derived DCs. (A) Monocytes cultured in medium containing GM-CSF and IL-4 for 7 days were analyzed by flow cytometry with or without gating cells for the expression CD1a using isotype-matched control or specific Ab. (B) The expression of the indicated molecules on the surface of gated cells were analyzed using isotype-matched control (thin lines) and specific (thick lines) Abs. The percentages and mean fluorescence intensity values of cells expressing the indicated molecules are shown. The data shown are representative of 3 experiments.

suspension by running through a nylon wool column. Varying numbers of DCs infected with EV71 or UV-inactivated EV71 for 24 hrs were cocultured with T cells (1×10^5) in 100 μl of medium. After 3 days, 50 μl of medium was added to the cultures. T-cell proliferation was measured by adding [^{3}H] thymidine into the cultures during the last 18 hrs of the 5-day incubation period, and the incorporated radioactivity was detected by a Beckman Coulter LS6500 scintillation counter.

Statistical Analysis. Data are expressed as mean ± SE values. Student's *t* test was used for statistical comparison. All *P* values are for 2-tailed significance tests. $P < 0.05$ was considered significant.

Results

Human Immature DCs Are Permissive to EV71 Infection. Human DCs, especially immature DCs, are highly specialized and efficient in uptaking and processing antigens and have been shown to support the growth of several viruses, including poliovirus and echovirus (20, 21, 24–30). Thus, we investigated the infection of human immature DCs by EV71. We prepared immature DCs by culturing monocytes purified from peripheral blood with cytokines, GM-CSF, and IL-4. Flow cytometric analysis with or without gating cells showed that >90% of resulting cells were CD1a$^+$ (Fig. 1A). Further analysis showed that

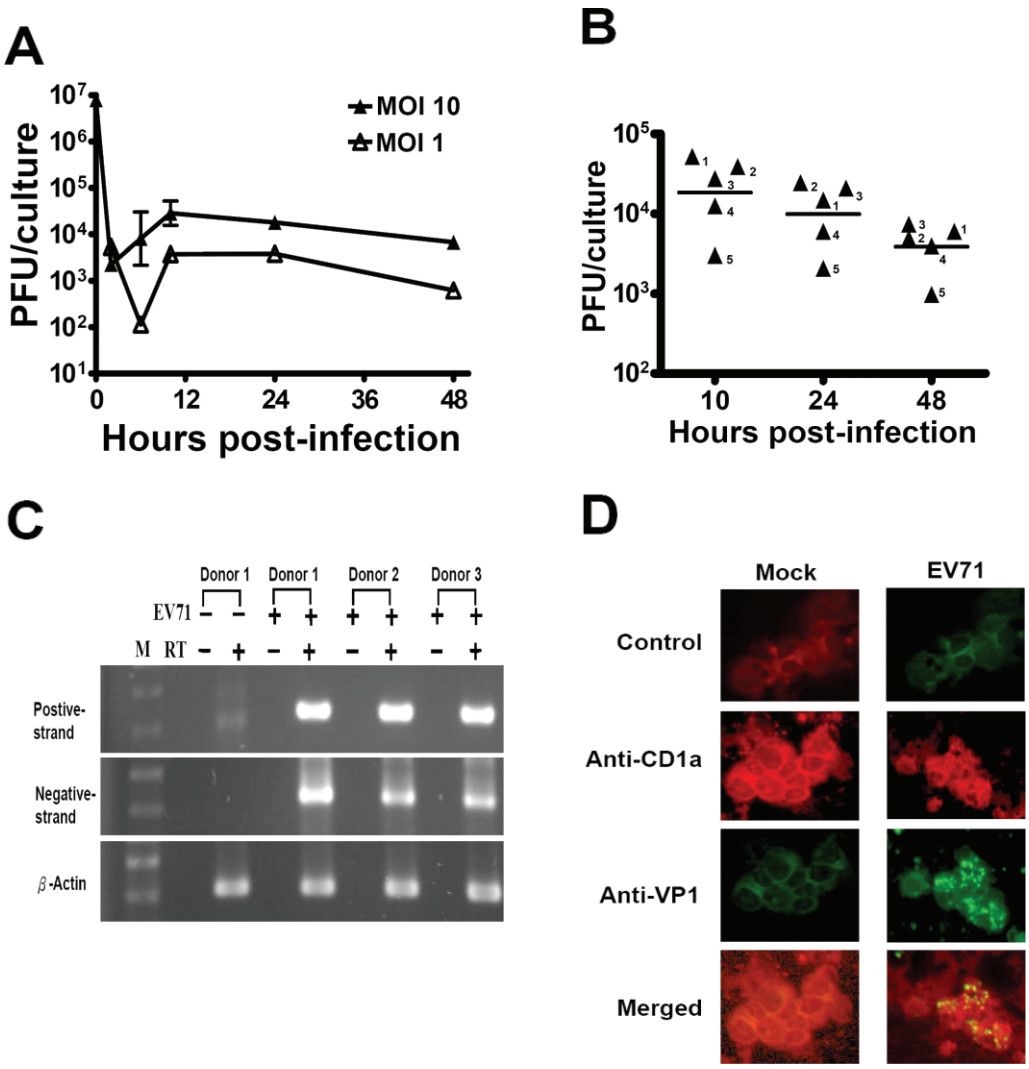

Figure 2. EV71 infection of immature DCs. (A) DCs were infected with EV71 (MOI 10 or 1), and culture supernatants were collected at the indicated times after infection to determine viral titers. Each point represents the mean ± SE values of 3 samples per time point titrated in duplicate. (B) The viral titers in the DCs cultured from different donors at the indicated times after infection (MOI 10) are shown. The numbers adjacent to symbols designate individual donors, and the bars represent the mean values for each group. (C) DCs cultured from Donors 1–3 were mocked infected (-) or infected (+) with virus (MOI 10) for 24 hrs. Total RNA was isolated from the cells and incubated with (+) or without (-) RT before PCR amplification. The presence of positive- and negative-strand RNA genomes in the cells is shown. β-actin serves as an internal control. M, DNA marker. (D) DCs were mocked infected (Mock) or infected (EV71) with virus (MOI 10) before staining with the indicated Abs. Images of cells stained with anti-CD1a and anti-VP1 Abs are merged.

$CD1a^+$ cells expressed CD80, CD86, MHC I, and MHC II, but not CD83 and CD14, on the surface of gated cells (Fig. 1B), demonstrating that they were immature DCs. We infected these DCs with a clinical isolate of EV71 (MOI = 10 or 1) and harvested culture supernatants to determine viral titers. The viral titers detected in the supernatants of cultures infected with a MOI of 10 increased from 2 to 10 hrs after infection and then declined afterward (Fig. 2A). The viral titers detected in the supernatants of cultures infected with a MOI of 1 increased from 2 to 24 hrs after infection and then declined afterward with a peak titer >9-fold lower than that detected in the supernatants of cultures infected with a MOI of 10. We chose the MOI of 10 for further experiments, because DCs produced more virus with this inoculum.

A previous report found that DCs efficiently supported dengue virus replication in the absence of feeding cytokines, GM-CSF, and IL-4 during infection (26). We also tested EV71 grown in the absence of feeding cytokines and found comparable titers with or without cytokines. We therefore conducted experiments without supplementing cytokines during EV71 infection to minimize the effects of cytokines. We next confirmed the productive infection of DCs by EV71 using DCs derived from more donors. Figure 2B shows that the DCs cultured from all 5 donors produced virus, although the viral titers yielded by DCs from different donors varied.

EV71, a virus with a positive-strand RNA genome, produces negative-strand RNA genomes during replication. Thus, the presence of negative-strand RNA genomes in

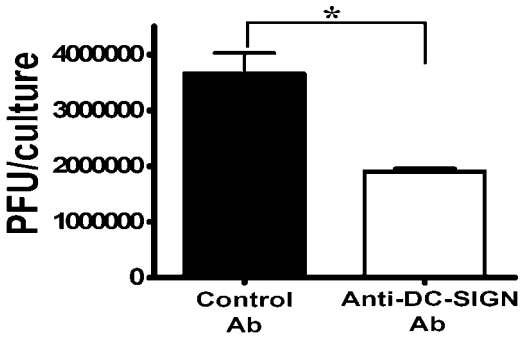

Figure 3. DC-SIGN mediates the entry of EV71 into DCs. The culture supernatants of infected DCs treated with control or anti–DC-SIGN Ab were harvested 24 hrs after infection to determine viral titers. The data shown are the mean ± SE values of 3 experiments. *, $P < 0.05$ by Student's *t* test.

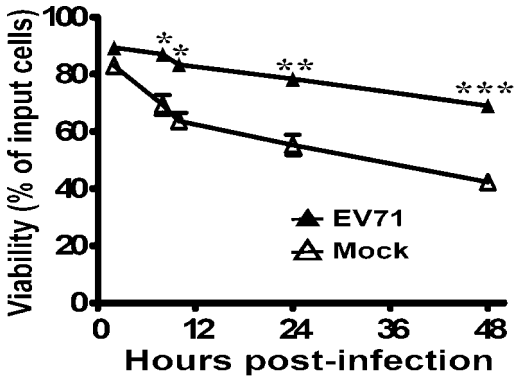

Figure 4. EV71 infection increases the viability of DCs. The viability of DCs mocked-infected (Mock) or infected with EV71 (EV71) were determined at the indicated times after infection by trypan blue exclusion assay. The data shown are the mean ± SE values of 3 experiments. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ compared with mock-infected group by Student's *t* test.

infected DCs serves as an index for viral replication. RT-PCR analysis detected both positive- and negative-strand RNA genomes in the infected DCs cultured from 3 different donors (Donors 1–3) but failed to detect either positive- or negative-strand viral genomes in the mock-infected DCs cultured from Donor 1 (Fig. 2C). In addition, immunofluorescence staining analysis using Abs to stain the molecule expressed in DCs, CD1a, or the viral capsid protein, VP1, showed that infected, but not mock-infected, DCs expressed VP1 (Fig. 2D). These results collectively show that immature DCs produce EV71 and express viral antigen after infection.

DC-SIGN Partially Mediates EV71 Entry into Immature DCs. DC-SIGN is a C-type lectin specifically expressed on immature DCs (27) and functions as a pattern recognition receptor for several viruses (21, 27, 28). We next investigated whether DC-SIGN also mediates the entry of EV71 into DCs by using an Ab to block DC-SIGN before infection of DCs with virus. The mean viral titer in the culture supernatants of infected DCs treated with anti–DC-SIGN Ab was reduced by about 50%, compared with that treated with control Ab (Fig. 3), suggesting that EV71 entry into DC is partially through DC-SIGN.

EV71 Infection Increases the Viability, Activation, and Cytokine Release of DCs. Viral infections have been shown to affect the viability, activation, and cytokine responses of DCs (20, 21, 24–26). Thus, we first investigated the effect of EV71 infection on the viability of DCs using trypan blue exclusion assay. Our results showed that mock-infected DCs lost viability gradually, with a survival rate of 42% after culture for 48 hrs (Fig. 4). Interestingly, infected DCs also lost viability but at a rate significantly slower than that of mock-infected DCs ($P < 0.05$ by Student's *t* test), with a survival rate of 69% after culturing for 48 hrs.

We also investigated the effect of EV71 infection on DC activation by determining the expression of co-stimulatory molecules, CD80 and CD86, and antigen presenting molecules, MHC I and II. Flow cytometric analysis of gated cells showed that EV71 infection increased the percentages of DCs expressing CD80, CD86, MHC I, and MHC II by 21, 23, 21, and 5%, respectively (Fig. 5). In addition, EV71 infection increased the mean fluorescence intensities of CD80, CD86, MHC I, and MHC II expressed on DCs by about 1.7-, 1.2-, 2.0-, and 2.1-fold, respectively. These results collectively show that EV71 infection activates DCs.

DCs secrete cytokines once they are activated. Among the cytokines secreted by DCs, IL-6, TNF-α, and IFN-γ levels are elevated, particularly in the sera and cerebrospinal fluid of EV71-infected patients with fatal symptoms (9, 15–18, 26). Thus, we harvested the culture supernatants from mock-infected or infected DCs 24 hrs after infection to measure these cytokines using ELISA kits. Our results showed that EV71 infection significantly increased the release of IL-6, IL-12, and TNF-α from DCs by 8.6-, 3.9-, and 3.3-fold, respectively (Fig. 6; $P < 0.05$ by Student's *t* test). However, EV71 infection failed to increase IFN-γ release from DCs.

EV71 Infection Enhances DCs to Stimulate T-Cell Proliferation. Infected DCs expressed viral antigen and were activated, so they should be able to prime T cells. We therefore examined the ability of infected DCs to prime naïve allogeneic T cells using mixed-lymphocyte reaction assay. Our results showed that infected DCs, but not mock-infected DCs or DCs infected with UV-inactivated virus, stimulated T-cell proliferation in a manner dependent on the number of DCs (Fig. 7).

Discussion

In this report, we show for the first time that EV71 can productively infect human DCs and express viral antigen. EV71 entry into DCs is partially through DC-SIGN. Further analyses revealed that EV71 increases the viability, activation, cytokine release, and T-cell priming activity of DCs.

Human immature DCs are shown to support the growth of several viruses (20, 21, 24–28). Some viruses, like herpes simplex virus, are shown to productively infect immature

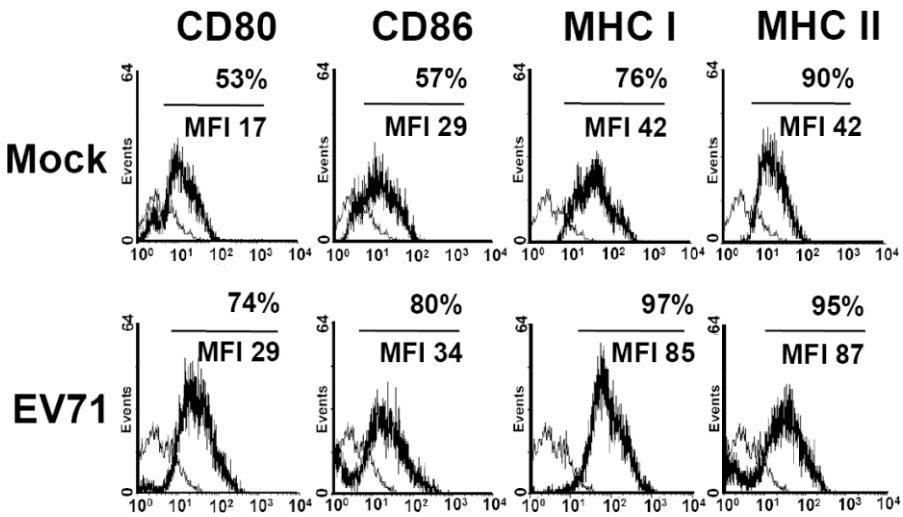

Figure 5. EV71 infection increases DC activation. DCs mocked-infected (Mock) or infected with EV71 (EV71) were analyzed using flow cytometry by gating cells for the expression of CD80 and CD86 24 hrs after infection and the expression of MHC I and MHC I 10 hrs after infection using isotype-matched control (thin lines) and specific (thick lines) Abs. The percentages and mean fluorescence intensity values of the cells expressing the indicated molecules are shown. The data shown are representative of 3 experiments.

DCs but not monocytes and mature DCs (24). Thus, we first tested the infection of immature DCs by EV71. We have also investigated the infection of monocytes and mature DCs by EV71 and found that these 2 types of cells produced slightly less and comparable amounts of virus, respectively, when compared with immature DCs (data not shown, see supplementary Fig. S1, available in the online version). These results suggest that EV71 is able to infect both immature and mature DCs. However, EV71 fails to alter the activation (CD80, CD86, MHC I, and MHC II expression) of mature DCs. We therefore focus on the infection of immature DCs by EV71 in the present study. Although EV71 increases the activation and T-cell priming activity of immature DCs, it fails to induce the maturation (CD83 expression) of immature DCs in a way similar to hantavirus (25). More signals are needed to induce the maturation of EV71-infected immature DCs. During infection, signals from damaged tissues trigger the migration of DCs into the draining lymphoid organs. In this process, DCs mature and become the most potent antigen-presenting cells. In EV71-infected patients, immature DCs might become mature and potent antigen-presenting cells during migration. Alternatively, both immature and mature DCs might be able to prime T cells in patients.

Among enteroviruses, poliovirus and echovirus, but not coxsackievirus B, are shown to productively infect human immature DCs (20, 21). Here we add EV71 to the list. However, what follows are some differences in the infection of DCs by these enteroviruses. (i) DC-SIGN mediates the entry of EV71 but not echovirus (21). The receptor for EV71

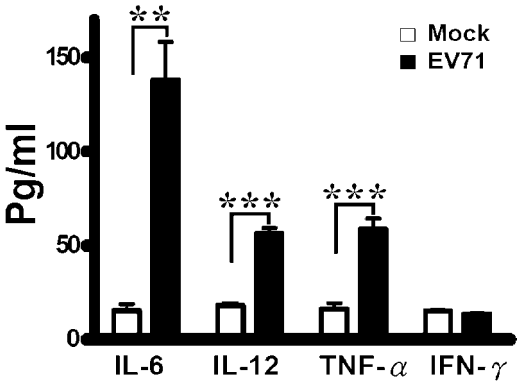

Figure 6. EV71 infection increases cytokine release from DCs. The culture supernatants of DCs mocked infected (Mock) or infected with EV71 (EV71) were harvested 24 hrs after infection to measure the indicated cytokines by ELISA. The data shown are the mean ± SE values of 3 experiments. **, $P < 0.01$ and ***, $P < 0.001$ by Student's *t* test.

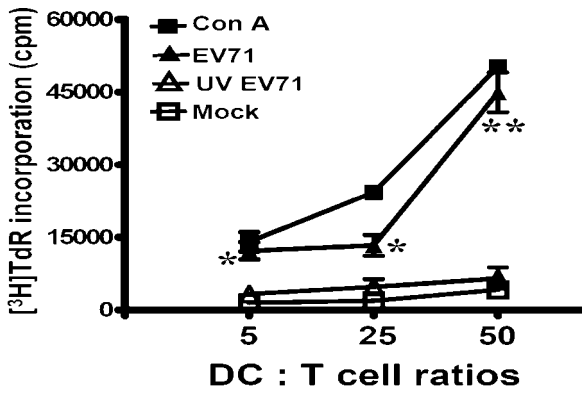

Figure 7. EV71 infection enhances DCs to stimulate T-cell proliferation. DCs mocked infected (Mock) or infected with EV71 (EV71) or UV-inactivated EV71 (UV EV71) for 24 hrs were cocultured with naïve allogenic T cells at the indicated ratios for 5 days to determine T-cell proliferation by [^{3}H] thymidine incorporation method. The proliferation of T cells treated with Con A (Con A) serves as a positive control. The data shown are the mean ± SE values of 3 experiments. *, $P < 0.05$ and **, $P < 0.01$ compared with mock-infected group by Student's *t* test.

remains elusive. Although our study is the first one to show that DC-SIGN mediates EV71 entry, some other receptor must exist, because DC-SIGN only partially mediates EV71 entry and other cells permissive to EV71, such as neuronal cells (31), normally do not express DC-SIGN. (ii) EV71 increases the viability of DCs, but coxsackievirus B does not affect the viability of DCs, and poliovirus and echovirus reduce the viability of DCs (20, 21). Absence of feeding cytokines has been shown to induce apoptosis in DCs (26). Dengue virus is shown to prevent caspase-dependent apoptosis in infected human mast cells (32). Further studies are needed to investigate whether EV7 increases the viability of DCs by preventing apoptosis. (iii) EV71 activates DCs, but poliovirus as well as coxsackievirus B do not activate DCs, and echovirus slightly reduces the activation of DCs by decreasing MHC I and II expression (20, 21). (iv) EV71 enhances DCs to prime T cells, but poliovirus does not affect the recognition of DCs by T cells (20). (v) EV71, but not echovirus, increases the cytokine release from DCs (21). All these results collectively show that the consequences regarding the infection of human DCs by enteroviruses vary and are difficult to predict.

In the present human study, EV71 enhances DCs to stimulate T cells and to release cytokines, such as IL-6. In mice, both T cells and IL-6 are shown to reduce mortality of infected mice by reducing tissue viral loads (19 and data not shown, see supplementary Fig. S2, available in the online version). We therefore believe that EV71 is very likely to enhance human DCs to elicit protective immunity *in vivo*.

Although EV71 infection has become a new threat to global public health in the past decade (6), the limited knowledge of EV71 pathogenesis hinders the development of effective vaccines to control fatal EV71 outbreaks. The notion that immunopathology is the cause of deaths promotes the use of anti-inflammatory agents, such as corticosteroids, to treat severe cases (3, 14). Our present human DC study and mouse study showing that DCs elicit protective immunity against EV71 infection raise cautions regarding such practice. Our findings suggest the potential of using vaccines to control fatal EV71 outbreaks. Currently, there are no effective vaccines available for EV71 infection. Our mouse study has shown that an increase of cellular immunity, especially DCs, by Flt3 ligand pretreatment protects mice from EV71 infection by reducing tissue viral loads. In the future, it will be of interest to explore the potential of using Flt3 ligand for prophylactic treatment or vaccine adjuvant to control fatal EV71 infection.

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